Review

Clinical Cancer Research

Invasive Bladder Cancer: Genomic Insights and Therapeutic Promise

Jaegil Kim[1], Rehan Akbani[2], Chad J. Creighton[3], Seth P. Lerner[3], John N. Weinstein[2], Gad Getz[1,4], and David J. Kwiatkowski[1,5]

Abstract

Invasive bladder cancer, for which there have been few therapeutic advances in the past 20 years, is a significant medical problem associated with metastatic disease and frequent mortality. Although previous studies had identified many genetic alterations in invasive bladder cancer, recent genome-wide studies have provided a more comprehensive view. Here, we review those recent findings and suggest therapeutic strategies. Bladder cancer has a high mutation rate, exceeded only by lung cancer and melanoma. About 65% of all mutations are due to APOBEC-mediated mutagenesis. There is a high frequency of mutations and/or genomic amplification or deletion events that affect many of the canonical signaling pathways involved in cancer development: cell cycle, receptor tyrosine kinase, RAS, and PI-3-kinase/mTOR. In addition, mutations in chromatin-modifying genes are unusually frequent in comparison with other cancers, and mutation or amplification of transcription factors is also common. Expression clustering analyses organize bladder cancers into four principal groups, which can be characterized as luminal, immune undifferentiated, luminal immune, and basal. The four groups show markedly different expression patterns for urothelial differentiation (keratins and uroplakins) and immunity genes (CD274 and CTLA4), among others. These observations suggest numerous therapeutic opportunities, including kinase inhibitors and antibody therapies for genes in the canonical signaling pathways, histone deacetylase inhibitors and novel molecules for chromatin gene mutations, and immune therapies, which should be targeted to specific patients based on genomic profiling of their cancers. *Clin Cancer Res; 21(20); 4514–24. ©2015 AACR.*

Disclosure of Potential Conflicts of Interest

S.P. Lerner reports receiving commercial research grants from Endo Pharmaceuticals and FKD Therapies, and is a consultant/advisory board member for BioCancell, Dendreon (uncompensated), Genentech, and OncoGenex Pharmaceuticals. No potential conflicts of interest were disclosed by the other authors.

Editor's Disclosures

The following editor(s) reported relevant financial relationships: J.R. Grandis—None.

CME Staff Planners' Disclosures

The members of the planning committee have no real or apparent conflicts of interest to disclose.

Learning Objectives

Upon completion of this activity, the participant should have a better understanding of the following aspects of bladder cancer: the genes commonly mutated and/or amplified, the mechanisms of mutation in bladder cancer, the classification of different bladder cancer types based upon expression profiles, and the genes whose mutations have potential for targeted therapy.

Acknowledgment of Financial or Other Support

This activity does not receive commercial support.

Introduction

Bladder cancer is a major cause of morbidity and mortality worldwide, with about 380,000 new cases and 150,000 deaths per year (1). It is notable among the common cancers in that both preinvasive and invasive forms of the disease are commonly diagnosed. Non–muscle-invasive bladder cancer (NMIBC), in which the smooth muscle layer surrounding the bladder is not invaded by tumor, accounts for about 80% of all bladder cancer diagnoses (1). NMIBCs (Ta and T1) include both low- and high-grade papillary tumors, and carcinoma *in situ*, a flat high-grade

[1]The Eli and Edythe L. Broad Institute of Massachusetts Institute of Technology and Harvard University, Cambridge, Massachusetts. [2]University of Texas MD Anderson Cancer Center, Houston, Texas. [3]Baylor College of Medicine, Houston, Texas. [4]Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts. [5]Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts.

Corresponding Authors: David J. Kwiatkowski, Brigham and Women's Hospital, 1 Blackfan Circle, Boston, MA 02115. Phone: 617-355-9005; Fax: 617-355-9016; E-mail: dk@rics.bwh.harvard.edu; and Jaegil Kim, The Eli and Edythe L. Broad Institute of Massachusetts Institute of Technology and Harvard University, 75 Ames Street, Cambridge, MA 02139. Phone: 617-714-8655; Fax: 617-800-1758; E-mail: jaegil@broadinstitute.org

doi: 10.1158/1078-0432.CCR-14-1215

Figure 1.
Mutation signatures in 238 muscle-invasive TCGA bladder cancers. A, Bayesian NMF (17) was used to identify five patterns of mutation that occur in bladder cancer genomes. Two of them match the APOBEC pattern, TCW -> TTW or TGW. The uppermost signature, APOBEC1, consists of both C>T and C>G mutations, whereas the next, APOBEC2, consists of only C>T mutations. The third mutation signature is that of CpG > TpG, the fourth is a POLE signature, and the fifth signature identified by NMF analysis is of unknown origin. The *y* axis gives the number of mutations of each type at each specific sequence. B, graph of the total number of mutations associated with five mutation signatures (top) and relative proportion of mutation types (bottom) seen in each TCGA bladder cancer sample.

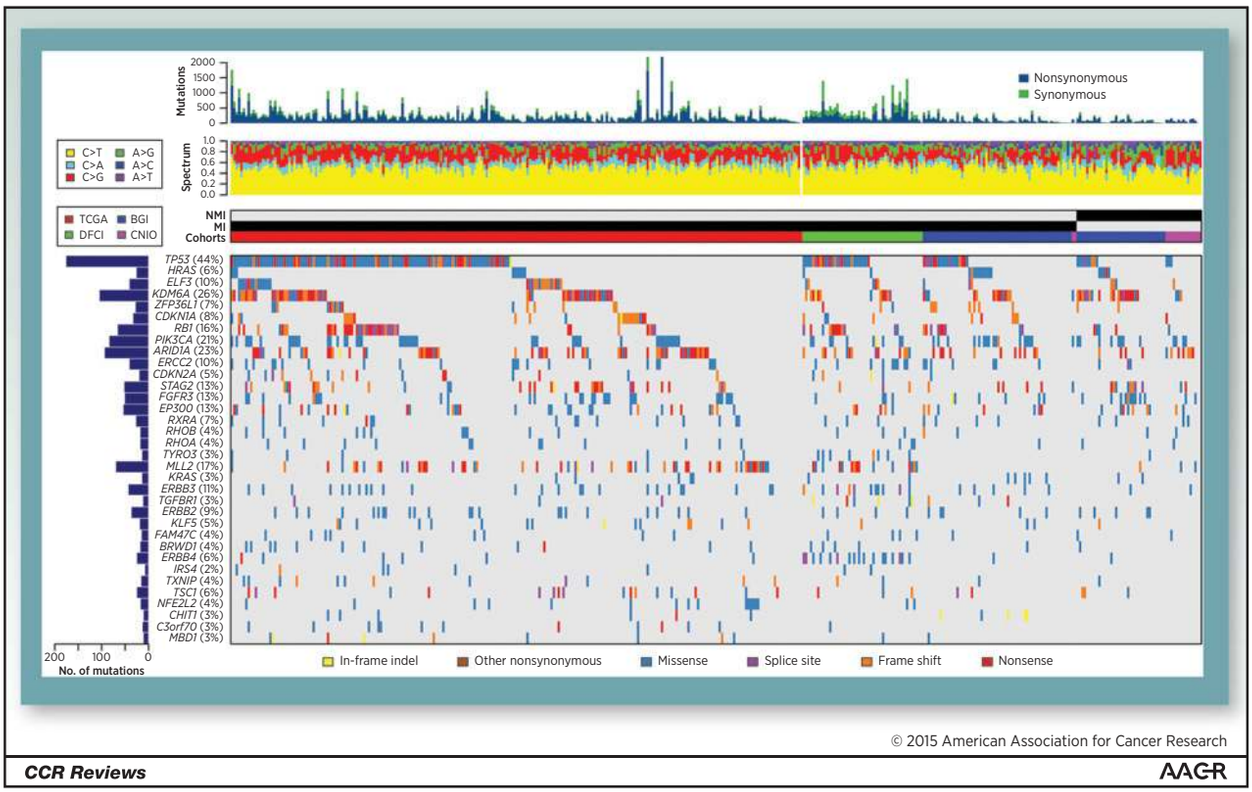

Figure 2.
Significantly mutated genes (SMG) identified in 404 cases of bladder cancer. Mutation data used were from TCGA (238 invasive cases; ref. 10), the Beijing Genomics Institute (62 invasive cases and 37 NMIBC: 28 T1N0, 2 T1bN0, 1 T1Nx, 6 TaN0; ref. 11), the CNIO (Spanish National Cancer Research Centre; 2 invasive and 15 NMIBC: 3 TaG1, 2 TaG2, 1 TaG3, 3 T1G2, 6 T1G3; ref. 12), and the DFCI/Broad (50 invasive cases; ref. 13). Sequentially from top to bottom: mutation rate, mutation spectrum, non–muscle-invasive (NMI) versus muscle-invasive (MI), source of data, and genes with statistically significant levels of mutation (MutSig 2CV; ref. 19, FDR < 0.1) sorted by *q* value are shown. Colors indicate different mutation types, shown at bottom. The total number of mutations and the percent of samples with mutation in each gene are shown at left. The CNIO data were not included in MutSig analysis to identify SMGs. Mutations seen at allele fraction $\leq$ 5% were not included. Five genes (*FAM82A2*, *STK39*, *ATP8P2*, *ZNF83*, and *GLT6D1*) identified by Mutsig were also deleted due to suspicious mutation patterns. Note that bar plots at the top are truncated for a few cancers.

tumor. NMIBC treatment consists of intravesical chemo- or immunotherapy and requires regular cystoscopic monitoring for early detection of recurrence and/or progression to invasive disease. Muscle-invasive bladder cancer, hereafter termed "invasive bladder cancer," is characterized by a high risk of metastases to regional pelvic lymph nodes and visceral sites, and is usually incurable despite systemic chemotherapy. Unfortunately, treatment of invasive bladder cancer has progressed little in the past two decades (2).

Past studies have identified multiple genes as commonly mutated in bladder cancer, including *TP53* (3), *RB1* (4), *TSC1* (5), *FGFR3* (6), and *PIK3CA* (7, 8). Many genomic regions of gain and loss have also been identified (1, 9).

A comprehensive review of the molecular pathogenesis of bladder cancer was recently published (1). Here, we focus on insights derived from the NIH NCI TCGA bladder cancer program (10) and other recent genome-wide analyses that include whole-exome sequencing (11–13).

High Mutation Rate in Bladder Cancer due to APOBEC-Type Mutagenesis

The Cancer Genome Atlas (TCGA) analysis of 130 invasive bladder cancers revealed a relatively high rate of mutation, a mean of 7.7, and median of 5.5 per Mb within coding regions, amounting to 302 protein-coding mutations per cancer (10). Lung adenocarcinoma, lung squamous cell carcinoma, and melanoma are the only major cancers studied by TCGA that have higher mutation rates. For those cancers, the causes are thought to be cigarette carcinogen mutagenesis (lung cancer) and sunlight UV mutagenesis (melanoma; ref. 14). Unexpectedly, the association between smoking history and mutation rate or mutation spectrum in TCGA cohort was rather weak (10), despite the known epidemiologic association between cigarette smoking and bladder cancer. In TCGA data, many mutations seen in bladder cancer were TCW -> TTW or TGW changes (nucleotide subject to change is underlined, W = A/T), a class of mutation probably mediated by one of the DNA cytosine deaminases in the APOBEC gene family (15, 16).

To examine mutational categories and processes in greater detail, we performed Bayesian non-negative matrix factorization (Bayesian-NMF) analysis (ref. 17; note that ref. 17 describes the original algorithm; full details of the method and its implementation will be described elsewhere) of the mutations stratified by 96 tri-nucleotide contexts in 238 TCGA bladder cancer specimens (Fig. 1), which were

Table 1. Genes identified as being significantly mutated or subject to focal copy-number change in bladder cancer

Genes identified as significantly mutated

Gene	Category	Total # mutations	% Samples with mutations	# Mutations NMIBC	% NMIBC	# Mutations invasive	% invasive	Higher in NMIBC *P*	Higher in NMIBC *q*	Higher in invasive *P*	Higher in invasive *q*
TP53	Cell cycle	176	44%	12	22%	164	47%	0.9999	1	0.0004	0.0158
HRAS	RAS	26	6%	5	9%	21	6%	0.2577	1	0.8829	0.9586
ELF3	Transcription	41	10%	4	7%	37	11%	0.8305	1	0.3318	0.6532
KDM6A	Chromatin	105	26%	23	43%	82	23%	0.0032	0.1229	0.9988	0.9988
ZFP36L1	Transcription	28	7%	2	4%	26	7%	0.9122	1	0.2477	0.5945
CDKN1A	Cell cycle	33	8%	1	2%	32	9%	0.9929	1	0.0468	0.3332
RB1	Cell cycle	66	16%	5	9%	61	17%	0.9637	1	0.0894	0.4246
PIK3CA	PI3K–mTOR	84	21%	13	24%	71	20%	0.3162	1	0.7960	0.9452
ARID1A	Chromatin	94	23%	11	20%	83	24%	0.7588	1	0.3640	0.6587
ERCC2	DNA repair	41	10%	3	6%	38	11%	0.9354	1	0.1695	0.5482
CDKN2A	Cell cycle	20	5%	0	0%	20	6%	1	1	0.0526	0.3332
STAG2	Chr. segregation	52	13%	11	20%	41	12%	0.0658	0.8339	0.9713	0.9988
FGFR3	RTK	51	13%	12	22%	39	11%	0.0247	0.4692	0.9907	0.9988
EP300	Chromatin	54	13%	9	17%	45	13%	0.2819	1	0.8375	0.9543
RXRA	Transcription	27	7%	4	7%	23	7%	0.4997	1	0.7145	0.8776
RHOB	Migration	18	4%	2	4%	16	5%	0.7212	1	0.5589	0.8249
RHOA	Migration	18	4%	2	4%	16	5%	0.7212	1	0.5589	0.8249
TYRO3	RTK	14	3%	1	2%	13	4%	0.8705	1	0.4199	0.7253
MLL2	Chromatin	70	17%	3	6%	67	19%	0.9999	1	0.00704	0.1337
KRAS	RAS	14	3%	2	4%	12	3%	0.5801	1	0.7159	0.8776
ERBB3	RTK	43	11%	2	4%	41	12%	0.9877	1	0.0514	0.3332
TGFBR1	RTK	12	3%	0	0%	12	3%	1	1	0.1742	0.5482
ERBB2	RTK	37	9%	5	9%	32	9%	0.5695	1	0.6284	0.8776
KLF5	Transcription	19	5%	1	2%	18	5%	0.9388	1	0.2503	0.5945
FAM47C	Other	15	4%	2	4%	13	4%	0.6196	1	0.6764	0.8776
BRWD1	Chromatin	18	4%	1	2%	17	5%	0.9289	1	0.2788	0.6233
ERBB4	RTK	25	6%	1	2%	24	7%	0.9754	1	0.1262	0.5330
IRS4	RTK	8	2%	0	0%	8	2%	1	1	0.3139	0.6532
TXNIP	ROS regulation	16	4%	1	2%	15	4%	0.9039	1	0.3438	0.6532
TSC1	PI3K–mTOR	25	6%	3	6%	22	6%	0.6760	1	0.5644	0.8249
NFE2L2	Transcription	17	4%	0	0%	17	5%	1	1	0.0827	0.4246
CHIT1	Other	11	3%	0	0%	11	3%	1	1	0.2020	0.5482
C3orf70	Other	13	3%	0	0%	13	4%	1	1	0.1502	0.5482
MBD1	Chromatin	11	3%	0	0%	11	3%	1	1	0.2020	0.5482
*RAS**	RAS	40	10%	7	13%	33	9%	0.2753	1	0.8539	0.9543
*RHO**	Migration	36	9%	4	7%	32	9%	0.7396	1	0.4572	0.7554

Genes subject to focal copy-number change

Gene symbol	Category	Total # focal CN change	% Samples with focal CN change
ARID1A	Chromatin	12	5
BCL2L1	Apoptosis	24	10
BEND3	Chromatin	8	3
BIRC3	Apoptosis	10	4
CCND1	Cell cycle	26	11
CCNE1	Cell cycle	22	9
CDKN2A	Cell cycle	100	43
CREBBP	Chromatin	38	16
E2F3	Elongation factor	43	18
EGFR	RTK	17	7
ERBB2	RTK	12	5
CCSER1	Mitosis	36	15
FGFR3	RTK	10	4
FHIT	Fragile site	30	13
FOXQ1	Transcription	25	11
GDI2	Migration	20	9
IKZF2	Transcription	35	15
LRP1B	Migration	39	17
MDM2	Cell cycle	21	9
MYC	Transcription	31	13
MYCL	Transcription	13	6
NCOR1	Chromatin	57	24
PDE4D	cAMP	52	22

(Continued on the following page)

Table 1. Genes identified as being significantly mutated or subject to focal copy-number change in bladder cancer (Cont'd)

Genes subject to focal copy-number change			
Gene symbol	**Category**	**Total # focal CN change**	**% Samples with focal CN change**
PPARG	Transcription	34	14
PRKCI	Protein kinase C, iota	9	4
PTEN	PI3K–MTOR	30	13
PVRL4	Matrix interactions	40	17
RB1	Cell cycle	39	17
SOX4	Transcription	42	18
WWOX	Other	35	15
YWHAZ	14-3-3-zeta	51	22
ZNF703	Transcription	24	10

NOTE: Genes identified as significantly mutated in bladder cancer (Mutsig 2CV; ref. 20) from analysis of 404 bladder cancers (350 invasive and 54 non–muscle-invasive), as described in the Fig. 1 legend and text. The different colors in the "Category" column for genes identified as being significantly mutated indicate the different functional categories of genes with mutations. The green color in the other columns highlights those *P* values that are nominally statistically significant ($P < 0.05$), and those *q* values that are statistically significant ($q < 0.2$).

Bottom: Genes identified as involved in copy-number change, either amplification or deletion, as identified by GISTIC2.0 (21). The red color in the "Gene symbol" column for genes subject to copy-number change denotes amplification; blue denotes deletion. Again, the different colors in the "Category" column highlight the different functional categories of genes with mutations. The asterisk denotes union of mutations of the types indicated: RAS means *KRAS* or *HRAS* and RHO means *RHOA* or *RHOB*.

Abbreviations: chr. segregation, chromosome segregation; RTK, receptor tyrosine kinase.

downloaded from Broad GDAC firehose. While conventional NMF requires the number of signatures as an input, Bayesian-NMF automatically prunes away irrelevant components that do not contribute to explaining observed mutations and effectively determines the appropriate number of signatures and their sample-specific contributions. That analysis identified five distinct patterns of mutagenesis operating among 73,301 single-nucleotide variants (SNV) in 238 bladder cancers (Fig. 1A). Two are variations of the APOBEC mutation signature, one consisting of C>T mutations in the TCW context ("APOBEC2"—17% SNVs), and the other consisting of both C>T and C>G mutations in the consensus ("APOBEC1"—48% SNVs). In contrast with other signatures, the third common mutation pattern ("unknown") is relatively nonspecific in terms of site and context and had a broad spectrum of base changes. Eighteen percent of the SNVs were associated with this signature of uncertain origin. The fourth pattern is the well-known C>T transition at CpG sites ("C>T_CpG"—10% SNVs). Interestingly, one sample with an ultra-high mutator phenotype (> 4,000 SNVs) had a *POLE* (DNA polymerase epsilon catalytic subunit) mutation commonly seen in colon and endometrial cancers (P286R), and a predominance of C>A mutations at TCT and C>T mutations at TCG sites ("POLE"—8% SNVs). Figure 1B shows that the number of mutations is highly variable among individual bladder cancers, as is the mutation signature. Overall, the APOBEC mutation pattern, with APOBEC1 and APOBEC2 signatures, accounts for about 65% of all point mutations, and is predominant in cancers with high mutation burdens apart from the single POLE hypermutated sample. However, there are some cancers with APOBEC mutation signature contribution as low as 5% (Fig. 1B). APOBEC3B is expressed at relatively high levels in all bladder cancers (10), and may be the mediator of APOBEC signature mutations (18). Notably, independent analysis of a smaller dataset ($n = 30$), but including whole-genome sequencing data for 4 samples, indicated that there was a strong APOBEC signature in 37% of bladder cancer, medium in 28%, and weak in 37% (19).

Genes Commonly Mutated in Bladder Cancer

To identify genes that are statistically significantly mutated in bladder cancer, we combined mutation datasets from TCGA (238 invasive cases; ref. 10), the Beijing Genomics Institute [(BGI); 62 invasive cases and 37 NMIBC; ref. 11], the CNIO (Spanish National Cancer Research Centre; 2 invasive and 15 NMIBC; ref. 12), and the Dana-Farber Cancer Institute/Broad Institute (50 invasive cases; ref. 13). Thirty-four genes were identified as being significantly mutated using Mutsig 2CV (ref. 20; Fig. 2 and Table 1) on this combined set of cohorts (not including the CNIO cohort due to unavailability of synonymous mutations), with rates of mutation varying from a high of 44% in *TP53* to a low of 2% in *IRS4*. Many other large genes had rates of mutation as high as 11% [e.g., *CREBBP* (11%), *MLL3* (11%), *ATM* (9%), *NF1* (7%), and *FBXW7* (6%)], but were not identified as statistically significantly mutated since the number of mutations that are expected to be random events ("noise") grows in proportion to the size of a gene. Statistically significantly mutated genes grouped into several different categories (Table 1). Genes related to receptor tyrosine kinase function, including several kinases, were significantly mutated ($n = 7$), as were those involved in chromatin regulation ($n = 6$), transcription ($n = 5$), and cell-cycle regulation ($n = 4$). The list of genes is similar to that reported previously from analyses of individual datasets (10, 11). Many pairs of genes from the list showed patterns of co-occurrence of mutations, including *TP53* and *RB1*, *STAG2* and *FGFR3*, *MLL2* and *NFE2L2*, *KDM6A* and *FGFR3*, and *ERBB3* and *ERBB4*, all with $q < 0.003$ (Fisher exact test with FDR correction used for these and subsequent analyses). Only a few pairs showed patterns of mutual exclusivity, *TP53* and any *RAS* gene, and *RB1* and *FGFR3*, $q < 0.02$.

The combination of both NMIBC and invasive bladder cancers in this analysis enabled assessment of differences in mutation rate between the two (Table 1). *MLL2* mutation was seen at a much higher rate in invasive bladder cancer (19% vs. 6% in NMIBC, $P = 0.007$, $q = 0.13$), as was *TP53* mutation (47% in

Figure 3.

Expression clustering identifies four different types of bladder cancer. Unsupervised hierarchical clustering (27) was performed on 238 TCGA bladder cancers using RNA-Seq RSEM expression values for the 3,000 most variable genes. Mutation and copy-number change data were also available for the 238 samples. A, mutation rate and type, histologic subtype, smoking status, gender, and tumor stage are shown. Four clusters were identified: red (luminal), orange (luminal immune), blue (basal), and green (immune undifferentiated). Four samples without complete data were not included in the clustering and are shown in gray (right). B, genes with statistically significant levels of mutation, as identified in Fig. 3, and mutation rates > 10% are shown, with mutation types. C, genomic regions with statistically significant focal copy-number changes (GISTIC2.0; ref. 21) are shown; limited to deletions seen in > 15% of samples, and amplifications seen in ≥5% of samples. "Copy number" refers to absolute copy number. The asterisk indicates that the gene listed is one among many within an amplification peak. D, RNA expression levels for selected genes, chosen to reflect luminal versus basal differentiation, and for roles in the immune system, are expressed as fold change from the median value for all samples. Gene fonts are color coded to indicate gene class and correlate with expression subtypes. Note that bar plots at the top are truncated for a few cancers. SCN, somatic copy-number alterations.

invasive vs. 22% in NMIBC, $P = 0.0004$, $q = 0.016$). Mutations in *KDM6A* were seen more commonly in NMIBC (43% vs. 23% in invasive bladder cancer, $P = 0.0032$, $q = 0.12$). Many previous studies have investigated differences in mutation frequency between NMIBC and invasive bladder cancer (1). In past studies, *FGFR3* mutation was much more common in low-grade NMIBC than in invasive cancer (∼70% vs. ∼12%), whereas *TP53* mutation was much more common in invasive cancer than low-grade NMIBC (∼40% vs. ∼7%; ref. 1). A recent small series identified a higher rate of mutations in *KDM6A* in NMIBC (65% in 30 NMIBC vs. 33% in 18 MIBC), concordant with our findings, as well as a higher rate of mutations in *TP53* in MIBC (56% of 18) versus NMIBC (5% of 20; ref. 19; these data were not included in our pooled analysis due to lack of availability of the primary data). Our observations based on these pooled genome-wide studies support mutation in *TP53* as being a key factor differentiating invasive bladder cancer from noninvasive disease. However, differences in *FGFR3* mutation were not seen. The observed differences in *MLL2* and *KDM6A* mutation rates between the two stage groups of bladder cancer are relatively novel. They suggest that different chromatin gene mutations contribute to the two different stage groups. However, these observations may be due in part to differences in the histologic characteristics of the NMIBC, or in the patient populations pooled for this analysis, or technical factors in the NGS analysis, and further study is required.

Amplification, Deletion, and Other Genomic Events in Bladder Cancer

Many comprehensive studies have identified numerous genomic amplification and deletion events occurring in

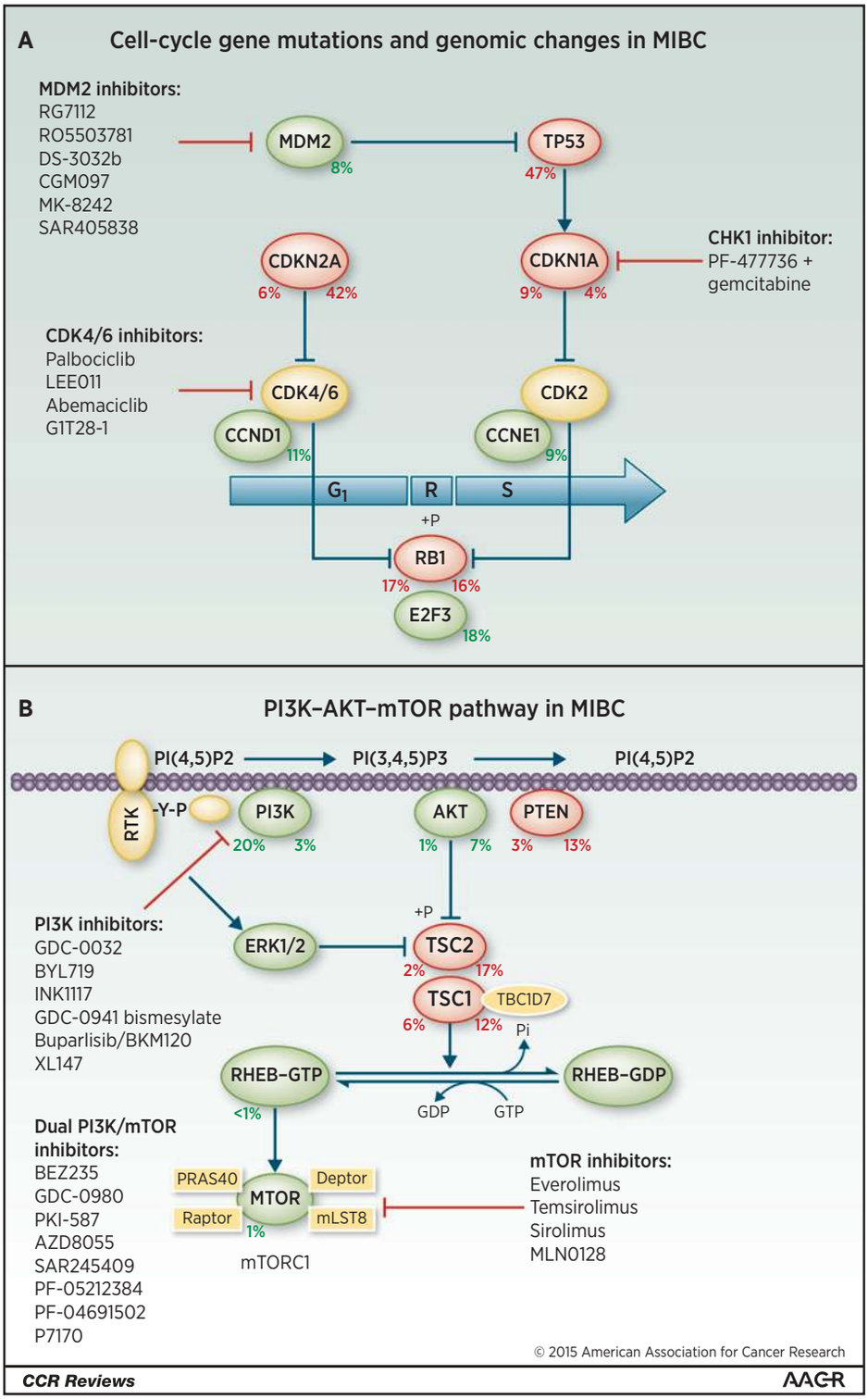

Figure 4.
Pathways, potential therapeutic targets, and possible inhibitors for invasive bladder cancer. Genes that drive growth or cancer progression are shown in green. Genes that are tumor suppressors and act to prevent growth or progression are shown in red. Beneath each gene symbol, the number on the left indicates the frequency of inactivating (red) or activating (green) mutation, the number on the right indicates the frequency of copy-number loss (red) or amplification (green). Classes of inhibitors and their targets are shown with blunt arrows indicating the components they inhibit. A, cell cycle. B, PI3K–AKT–mTOR pathway. (*Continued on the following page.*)

bladder cancer (1, 9). These findings were confirmed and extended in the recent TCGA analysis based upon Affymetrix SNP profiling and low-pass whole-genome sequencing, both analyzed by GISTIC (21). Thirteen genes were targets of focal deletion and 19 were targets of focal amplification (Table 1). The majority of those genes fall into the same categories as

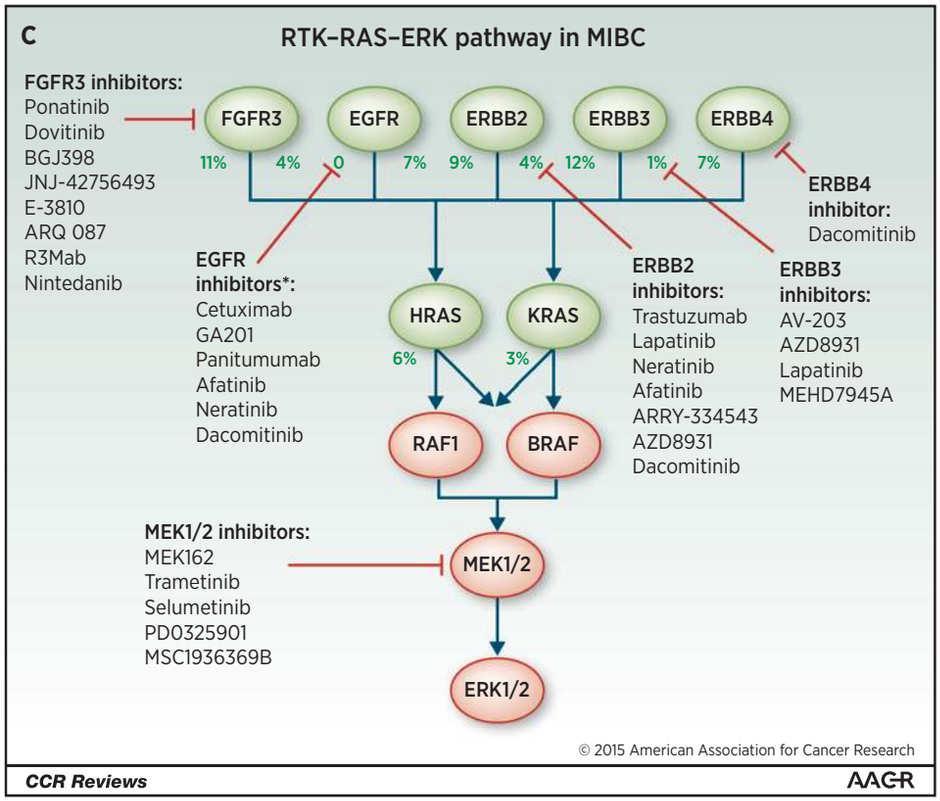

Figure 4.
(*Continued.*) C, RTK–RAS–ERK pathway (asterisk indicates not erlotinib).

those for which mutations are seen, including cell cycle, chromatin regulation, receptor tyrosine kinase signaling, and transcription.

In TCGA dataset, 3 (2%) invasive bladder cancers contained *FGFR3–TACC3* fusion sequences (11), a chromosomal translocation identified previously in bladder cancer (22). These fusion proteins are highly transforming. They have now been seen in multiple cancer types, and cancers bearing them may be especially sensitive to FGFR3 inhibitors. Four (3%) cancers had fusions involving *ERBB2* and various other genomic regions of uncertain functional significance (11).

Subsets of Bladder Cancer Based upon Expression Profiling

Several recent studies have performed comprehensive gene expression profiling analysis of high-grade or muscle-invasive bladder cancer and used unsupervised hierarchical clustering to define expression pattern subtypes (10, 23–26). Although the findings from those analyses have not been completely uniform, there is considerable similarity. The report by Sjodahl and colleagues identified five expression subtypes, Urobasal A and B, genomically unstable, squamous cell carcinoma-like (SCC-like), and infiltrated (referring to the presence of nontumor cells; ref. 26). A subtype termed "basal" was identified by all of the other studies (10, 24, 25) and is characterized by expression of keratins KRT5, KRT14, and KRT6A/B/C, as well as HES2 and MYC, indicative of a basal or stem cell phenotype, and is similar to the SCC-like subtype of Sjodahl and colleagues. The three later studies also identified a "luminal" expression subtype, so-called because of its similarity to breast cancer luminal subtypes, characterized by high expression of FGFR3, the uroplakin genes, KRT20, and transcription factors PPARG, GATA3, FOXA1, and RXRA. The luminal subtype was similar to the Urobasal A subtype in the Sjodahl study. Another subtype, p53-like, was also identified in one of these studies (24). All of these analyses for which prognostic information was available showed the basal subtype to be associated with poorer prognosis, and the luminal subtype to be associated with more favorable prognosis (23–26).

To examine patterns of expression in invasive bladder cancer in greater detail, we performed unsupervised hierarchical clustering (27) on 238 TCGA bladder cancers for which both RNA-Seq and whole-exome sequencing mutational analysis had been performed (Fig. 3). That analysis gave results similar to those published on the 131 samples (ref. 10; Rand index = 0.82), and identified four different subtypes, splitting the luminal and basal subtypes into two further subtypes each. Forty-one percent of the invasive cancers were in the luminal subtype (red, Fig. 3) with high expression of KRT20 and UPKs 2/1A/1B/3A, as well as moderate to high expression of multiple pertinent transcription factors (KLF5, PPARG, and GRHL5). The luminal subtype was enriched in male patients, papillary histology, and stage II tumors, and is similar to the previously identified luminal (10, 24, 25) and urobasal A (26) subtypes. Twenty-nine percent of the invasive bladder cancers were in the Basal subtype (blue, Fig. 3) with high expression of KRT14, KRT5, KRT6A/B, and KRT16, and low

expression of all uroplakins, consistent with a basal or undifferentiated cytokeratin expression pattern. Consistent with previous studies, the basal subtype expressed TP63, TP73, MYC, and EGFR, as well as TGM1 and SCEL, indicative of some degree of squamous differentiation (10, 24–26). The Basal subtype was enriched in female patients and nonpapillary histology, and also expressed many immune genes at intermediate and somewhat variable levels, including CTLA4 and CD274 (encodes PD-L1), suggestive of immune cell infiltration. Eleven percent of the cancers grouped into a novel subtype that we term immune undifferentiated (green, Fig. 3). Those cancers showed very low expression of luminal markers, with variable expression of basal cytokeratins, and relatively high-level expression of many immune genes, including CTLA4 and CD274, suggesting significant immune cell infiltration and possible immune evasion (see further below). Last, the luminal immune subtype (18%; orange, Fig. 3) was characterized by expression of luminal genes (cytokeratins and uroplakins), and intermediate expression of immune genes, and was enriched for Stage N+ tumors. The luminal subtype was enriched in cancers with mutations in *FGFR3* and amplification events involving *PVRL4* and *YWHAZ*, whereas the basal subtype was enriched in mutations in *NFE2L2* (all with $P < 0.02$ and $q < 0.2$; Fig. 3). Furthermore, both luminal immune and immune undifferentiated subtypes had a high level of expression of ZEB1, ZEB2, and TWIST1 characteristic of epithelial-to-mesenchymal transition (EMT).

Therapeutic Targets in Invasive Bladder Cancer

Those observations, along with continuing drug development in the pharmaceutical industry, have led to a large number of potential therapeutic opportunities for invasive bladder cancer.

First, the high frequency of mutations and genomic deletions affecting chromatin regulatory genes in bladder cancer, higher than in any other epithelial malignancy (11), suggests that therapies targeted at the effects of those mutations could be useful. Mocetinostat, an oral second-generation HDAC inhibitor, is currently being assessed in a clinical trial for invasive bladder cancers with mutations in either *EP300* or *CREBBP* (28). Further pharmaceutical development of agents that target those mutations is needed and is actively being pursued.

Second, mutations and genomic deletion or amplification events that affect the cell cycle are very common in bladder cancer. Those include alterations of *TP53* and the cyclin-dependent kinase inhibitors *CDKN1A* and *CDKN2A* (Fig. 4A). Both CDNK2A loss and amplification of cyclin D1 (gene symbol *CCND1*) can be targeted by agents in development that are CDK4/6 inhibitors, including palbociclib (29). MDM2, amplified in 8% of invasive bladder cancer, is also a therapeutic target of several drugs in development. *CDKN1A* mutation, although extremely rare in other cancer types, is seen in 14% invasive bladder cancer and occurs with concurrent *TP53* mutation about half the time (30). Concurrent loss of *CDKN1A* and *TP53* has been shown in cell line and mouse xenograft models to lead to marked sensitivity to combined treatment with gemcitabine and a CHK1 inhibitor, such as PF477736, suggesting potential clinical utility (30).

Third, the PI3K–AKT–mTOR pathway is commonly subject to mutation in invasive bladder cancer (Fig. 4B). Multiple agents are in clinical development to target PI3-kinase (gene name *PIK3CA*), one of the genes most commonly mutated in bladder cancer. Though less common than *PIK3CA* mutations, mutations in *TSC1* are well-known in bladder cancer (5), and they have been shown in at least some cases to lead to dramatic sensitivity to treatment with mTOR kinase inhibitors, such as everolimus (31). Further studies are under way to define the precise clinical and genetic characteristics of response to mTOR inhibition in bladder cancer.

Fourth, the extent of the receptor tyrosine kinase–RAS–ERK signaling pathway involvement in invasive bladder cancer has recently become much more evident (Fig. 4C). Both *FGFR3* and all the members of the ERBB family are affected by either activating mutations or amplification events (Fig. 4C). Drugs that target those genetic abnormalities are at various stages of clinical development, and arguably, FGFR3-activating mutations and gene fusions are the most promising targets among those genes. Clinical trials of FGFR3 kinase inhibitors against bladder and other cancers are ongoing (32).

Last, immune therapy has shown considerable promise for the treatment of invasive bladder cancer. A recent report indicated that one immunomodulatory treatment approach, use of the humanized anti–PD-L1 monoclonal antibody MPDL3280A, had significant activity in bladder cancer (33). Of those patients whose bladder tumors contained high amounts of tumor-infiltrating cells expressing PD-L1 as assessed by immunohistochemistry, 13 of 25 (52%) showed an objective response after 12 weeks on therapy, and the response was ongoing (33). These results build upon a large and growing body of evidence that immune evasion through cancer-induced immunosuppression, often through activation of immune checkpoints, is an important factor in cancer progression (34). For example, both cytotoxic T-lymphocyte associated antigen-4 (CTLA4) and programmed death-1 (PD-1) receptors expressed by T cells can be engaged by corresponding receptor molecules on cancer cells (e.g., PD-L1) or other immune cells to block lymphocyte activity directed at cancer cells (34). Hence, antibodies that block such interaction, directed at either of the interacting molecules, can interfere with cancer checkpoint blockade, leading to native immune cell attack on the cancer, and therefore, to clinical response. The relatively high level of immune gene expression by some bladder cancers, including CTLA4 and CD274 (encoding PD-L1; Fig. 3), is consistent with the hypothesis that a subset of bladder cancers is characterized by immune suppression, and will be sensitive to immune-modulatory therapy. Clinical trials of multiple immune therapy agents are in progress for bladder cancer (2, 28). Based upon our current analyses, it appears that the immune undifferentiated and basal subtypes of bladder cancer will be the most promising subtypes for immune checkpoint therapy (Fig. 3). However, further analysis is urgently needed so that these therapies can be applied with the most precision and effectiveness in bladder cancer.

Conclusions

Invasive bladder cancer is characterized by a high overall mutation rate, which appears to be explained mainly by APOBEC-

mediated mutagenesis. Both well-known and relatively novel cancer genes are commonly affected in invasive bladder cancer by mutation, genomic amplification/deletion, or both. Genes affected include those involved in transcription, chromatin regulation, receptor tyrosine kinase signaling, PI3K–mTOR signaling, RAS, and the cell cycle. Expression profiling studies are consistent in the identification of two main subtypes of bladder cancer, broadly definable as basal and luminal. Basal tumors are less differentiated, more aggressive, and more lethal; luminal tumors are more differentiated and show higher expression levels of uroplakins and FGFR3. Expression clustering reveals additional subtypes within the two main groups, and, quite significantly, the subtypes differ in immune gene expression and EMT marker expression.

The future is looking bright for therapeutic advances in bladder cancer. Promising targets and drugs in development that should be deployed in mutation- and expression-specific fashions, as per the "precision medicine" paradigm. Promising therapeutic agents directed against the cell cycle, receptor tyrosine kinase pathway, and PI3K–mTOR pathway mutations are in hand. Mutations in chromatin regulatory genes are promising targets for which further pharmaceutical development will be required. Immune checkpoint agents, already in the clinic, also show promise, and the expression/mutational subtypes defined above may aid both our preclinical and clinical progress with them.

Grant Support

R. Akbani and J.N. Weinstein are supported by the NCI of the NIH under award numbers CA143883 and P30CA016672, CPRIT RP130397, the Mary K. Chapman Foundation, and the Michael & Susan Dell Foundation. C.J. Creighton is supported by the NCI of the NIH under award numbers P30CA125123 and U24CA143843. D.J. Kwiatkowski is supported by the NCI of the NIH under award number P01CA120964.

Received May 12, 2014; revised June 3, 2015; accepted June 19, 2015; published online October 15, 2015.

References

1. Knowles MA, Hurst CD. Molecular biology of bladder cancer: new insights into pathogenesis and clinical diversity. Nat Rev Cancer 2015; 15:25–41.
2. Carballido EM, Rosenberg JE. Optimal treatment for metastatic bladder cancer. Curr Oncol Rep 2014;16:404.
3. Esrig D, Spruck CH 3rd, Nichols PW, Chaiwun B, Steven K, Groshen S, et al. p53 nuclear protein accumulation correlates with mutations in the p53 gene, tumor grade, and stage in bladder cancer. Am J Pathol 1993;143:1389–97.
4. Cote RJ, Dunn MD, Chatterjee SJ, Stein JP, Shi SR, Tran QC, et al. Elevated and absent pRb expression is associated with bladder cancer progression and has cooperative effects with p53. Cancer Res 1998;58:1090–4.
5. Hornigold N, Devlin J, Davies AM, Aveyard JS, Habuchi T, Knowles MA. Mutation of the 9q34 gene TSC1 in sporadic bladder cancer. Oncogene 1999;18:2657–61.
6. Cappellen D, De Oliveira C, Ricol D, de Medina S, Bourdin J, Sastre-Garau X, et al. Frequent activating mutations of FGFR3 in human bladder and cervix carcinomas. Nat Genet 1999;23:18–20.
7. Lopez-Knowles E, Hernandez S, Malats N, Kogevinas M, Lloreta J, Carrato A, et al. PIK3CA mutations are an early genetic alteration associated with FGFR3 mutations in superficial papillary bladder tumors. Cancer Res 2006;66:7401–4.
8. Platt FM, Hurst CD, Taylor CF, Gregory WM, Harnden P, Knowles MA. Spectrum of phosphatidylinositol 3-kinase pathway gene alterations in bladder cancer. Clin Cancer Res 2009;15:6008–17.
9. Goebell PJ, Knowles MA. Bladder cancer or bladder cancers? Genetically distinct malignant conditions of the urothelium. Urol Oncol 2010;28: 409–28.
10. Cancer Genome Atlas Research N. Comprehensive molecular characterization of urothelial bladder carcinoma. Nature 2014;507:315–22.
11. Guo G, Sun X, Chen C, Wu S, Huang P, Li Z, et al. Whole-genome and whole-exome sequencing of bladder cancer identifies frequent alterations in genes involved in sister chromatid cohesion and segregation. Nat Genet 2013;45:1459–63.
12. Balbas-Martinez C, Sagrera A, Carrillo-de-Santa-Pau E, Earl J, Marquez M, Vazquez M, et al. Recurrent inactivation of STAG2 in bladder cancer is not associated with aneuploidy. Nat Genet 2013;45:1464–9.
13. Van Allen EM, Mouw KW, Kim P, Iyer G, Wagle N, Al-Ahmadie H, et al. Somatic ERCC2 mutations correlate with cisplatin sensitivity in muscle-invasive urothelial carcinoma. Cancer Discov 2014;4: 1140–53.
14. Lawrence MS, Stojanov P, Polak P, Kryukov GV, Cibulskis K, Sivachenko A, et al. Mutational heterogeneity in cancer and the search for new cancer-associated genes. Nature 2013;499:214–8.
15. Nik-Zainal S, Alexandrov LB, Wedge DC, Van Loo P, Greenman CD, Raine K, et al. Mutational processes molding the genomes of 21 breast cancers. Cell 2012;149:979–93.
16. Roberts SA, Sterling J, Thompson C, Harris S, Mav D, Shah R, et al. Clustered mutations in yeast and in human cancers can arise from damaged long single-strand DNA regions. Mol Cell 2012;46: 424–35.
17. Tan VY, Fevotte C. Automatic relevance determination in nonnegative matrix factorization with the beta-divergence. IEEE Trans Pattern Anal Mach Intell 2013;35:1592–605.
18. Roberts SA, Gordenin DA. Hypermutation in human cancer genomes: footprints and mechanisms. Nat Rev Cancer 2014;14:786–800.
19. Konsavage WM Jr, Kyler SL, Rennoll SA, Jin G, Yochum GS. Wnt/beta-catenin signaling regulates Yes-associated protein (YAP) gene expression in colorectal carcinoma cells. J Biol Chem 2012;287: 11730–9.
20. Lawrence MS, Stojanov P, Mermel CH, Robinson JT, Garraway LA, Golub TR, et al. Discovery and saturation analysis of cancer genes across 21 tumour types. Nature 2014;505:495–501.
21. Zack TI, Schumacher SE, Carter SL, Cherniack AD, Saksena G, Tabak B, et al. Pan-cancer patterns of somatic copy number alteration. Nat Genet 2013; 45:1134–40.
22. Williams SV, Hurst CD, Knowles MA. Oncogenic FGFR3 gene fusions in bladder cancer. Hum Mol Genet 2013;22:795–803.
23. Volkmer JP, Sahoo D, Chin RK, Ho PL, Tang C, Kurtova AV, et al. Three differentiation states risk-stratify bladder cancer into distinct subtypes. Proc Natl Acad Sci U S A 2012;109:2078–83.
24. Choi W, Porten S, Kim S, Willis D, Plimack ER, Hoffman-Censits J, et al. Identification of distinct basal and luminal subtypes of muscle-invasive bladder cancer with different sensitivities to frontline chemotherapy. Cancer Cell 2014;25:152–65.
25. Damrauer JS, Hoadley KA, Chism DD, Fan C, Tiganelli CJ, Wobker SE, et al. Intrinsic subtypes of high-grade bladder cancer reflect the hallmarks of breast cancer biology. Proc Natl Acad Sci U S A 2014;111: 3110–5.
26. Sjodahl G, Lauss M, Lovgren K, Chebil G, Gudjonsson S, Veerla S, et al. A molecular taxonomy for urothelial carcinoma. Clin Cancer Res 2012; 18:3377–86.
27. Wilkerson MD, Hayes DN. ConsensusClusterPlus: a class discovery tool with confidence assessments and item tracking. Bioinformatics 2010;26: 1572–3.
28. Sonpavde G, Jones BS, Bellmunt J, Choueiri TK, Sternberg CN. Future directions and targeted therapies in bladder cancer. Hematol Oncol Clin North Amer 2015;29:361–76.

29. Rocca A, Farolfi A, Bravaccini S, Schirone A, Amadori D. Palbociclib (PD 0332991): targeting the cell cycle machinery in breast cancer. Expert Opin Pharmacother 2014;15:407–20.
30. Liu Y, Kwiatkowski DJ. Combined CDKN1A/TP53 mutation in bladder cancer is a therapeutic target. Mol Cancer Ther 2015;14: 174–82.
31. Iyer G, Hanrahan AJ, Milowsky MI, Al-Ahmadie H, Scott SN, Janakiraman M, et al. Genome sequencing identifies a basis for everolimus sensitivity. Science 2012;338:221.
32. Mazzola CR, Siddiqui KM, Billia M, Chin J. Dovitinib: rationale, preclinical and early clinical data in urothelial carcinoma of the bladder. Expert Opin Investig Drugs 2014;23:1553–62.
33. Powles T, Eder JP, Fine GD, Braiteh FS, Loriot Y, Cruz C, et al. MPDL3280A (anti-PD-L1) treatment leads to clinical activity in metastatic bladder cancer. Nature 2014;515:558–62.
34. Drake CG, Lipson EJ, Brahmer JR. Breathing new life into immunotherapy: review of melanoma, lung and kidney cancer. Nat Rev Clin Oncol 2014; 11:24–37.